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INTRODUCTION

Our goal is to increase the efficacy of T cell attack against breast cancer that has metastasized to the brain. This is a major clinical problem, and T cell therapy is well-suited to attack of tumor that may be difficult to image or access conventionally. The first task was to develop a suitable model: 1) We adapted methods for introducing tumor into the carotid artery, to favor delivery to the brain. 2) We modified the rat mammary carcinoma cell line, 13762 MAT BIII, so that it expresses the lacZ reporter gene product, E. coli b-galactosidase (b-gal)(*MATB/lacZ*). The b-gal permits unambiguous identification of even single tumor cells and serves as a defined tumor antigen. 3) We showed that after intra-carotid injection of MATB/lacZ, metastases do appear in the brain. The sites of metastatic tumor and time-to-illness are dose-dependent. The time-to-illness is sufficient for our goals. 4) Our therapy involves introducing activated, tumor-specific T cells into the blood and injecting immuno-activating cytokine (IFN- γ) into the brain. We developed fluorescence-labeled T cell lines that allow unambiguous identification of the responding T cells, and did other preparatory work. Now we are ready to test our proposed therapy. The model itself is of general interest.

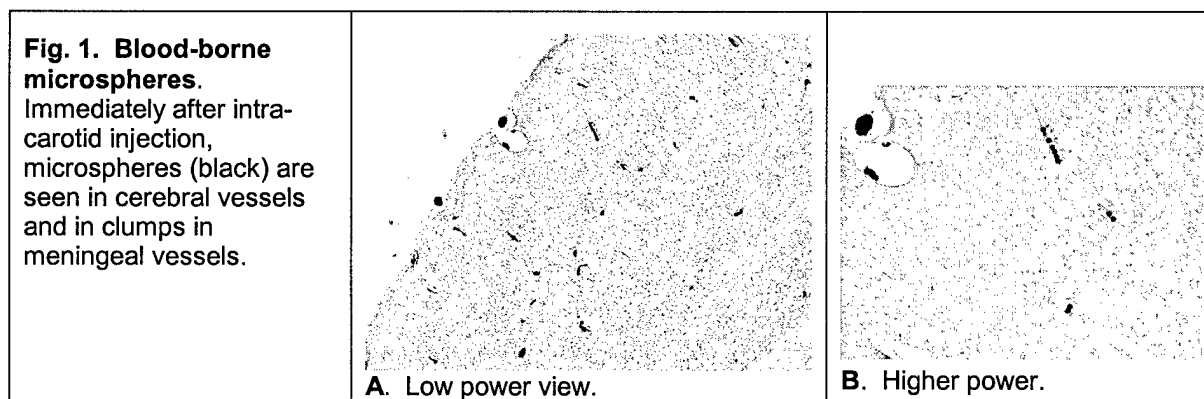
BODY OF REPORT: Summary of work done, with reference to original Statement of Work.**Task 1. Develop model for brain-metastasizing breast cancer (AIM 1A), months 1-6.**

Sub-tasks (summarized). Inject rat mammary carcinoma cell line into blood of syngeneic rats. Define spatial and temporal pattern of blood-borne metastases in the brain.

Exp. 1. Developing the injection methodology. We considered two ways of delivering tumor to the blood. Intra-carotid injections provide the most direct route to the brain. Intra-cardiac injections are technically simpler. In practice, we found that the intra-carotid injections do not present a technical problem, and our veterinary staff (Dr. Angie Warner) advises that they are safer for the rat. Therefore, we focused on intra-carotid injections.

We adapted commonly-used methods for exposing the carotid artery. To learn and adapt the methods for our use, we injected polystyrene microspheres as markers. We first tested microspheres of 50u and 10u diameter, then living cells (below). We compared different sizes and materials for needles and tubing, variations in the number of side-branches that were tied off, and use of a micro-injection pump vs. injection by hand.

Results. Among the variations tried, temporarily clamping the external carotid artery and injecting by hand with a 30.5g needle into the internal carotid artery via the common carotid artery was the most rapid and gave the highest yield of microspheres inside the brain. (For 10 u diameter micro-spheres, the yield was 30-40% in 5/6 rats, evaluated immediately after injection). Immediately after injection, the microspheres are most common in blood vessels of the cerebral cortex on the injection side (fig. 1). This is the pattern that would be predicted from the blood flow (Scremin 95).



Conclusion. We developed an intracarotid injection method that is appropriate for delivering tumor-sized objects to the brain, and confirmed that it delivers inert beads to the brain in the expected pattern.

Exp. 2. Preparation and detection of tumor cell line. 13762 MAT BIII is a highly metastatic variant of the widely-used 13762 rat mammary carcinoma cell line. The original tumor is from a CDF (CD Fischer) rat, the strain we used in all of our studies. We adapted the cell line and detection methods, as described below.

a. To better visualize individual metastatic tumor cells, the 13762 MAT BIII cell line was made to express the lacZ reporter gene product, E. Coli b-galactosidase, using our previous methods (Lampson et al 92). The enzyme is constitutively expressed as a cytoplasmic protein, one that is readily detected after histochemical stain for b-gal. The histochemical stain can detect b-gal+ cells in culture wells and in tissue sections (Lampson et al 92).

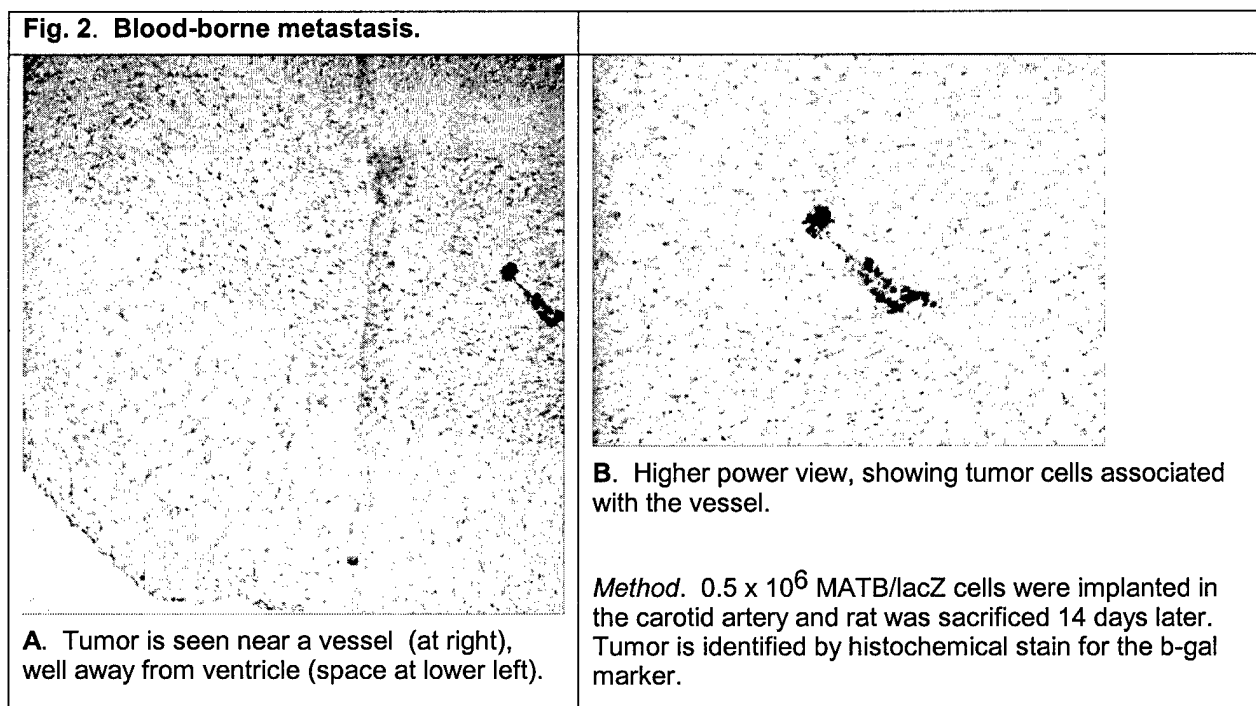
To prepare the lacZ-expressing cell line, 13762 MAT BIII cells were infected with a retroviral vector containing the lacZ gene, drug-selected, then cloned at limiting dilution (Lampson et al 92). Most recently, we re-cloned the line and selected a subclone with strong constitutive lacZ expression. Below, this cell line is referred to as *MATB/lacZ*. This cell line was used in all of the studies described below, and retains its strong b-gal expression (fig. 2, next page).

Conclusion. The *MATB/lacZ* cell line constitutively expresses the b-gal marker. This allows detection of single tumor cells in tissue sections. Moreover, the b-gal protein serves as a well-defined tumor antigen (Lampson et al 93).

b. In our previous work (Lampson et al 92, 93), we used a widely-used histochemical stain for b-gal. The main potential source of false positives is from mammalian b-gal in phagocytes, since b-gal is a lysosomal enzyme. The two activities can be separated because the mammalian enzyme and the *E. coli* enzyme have different pH optima. However, the commonly used method sometimes shows weak b-gal activity in phagocytes.

In this project, we wanted to be able to evaluate tumor areas that are rich in phagocytes, including regions of the brain we had not previously stressed (such as the choroid plexus), and organs such as lung and lymph node, where the tumor can also grow (see below).

Conclusion. The MATB/lacZ tumor can be unambiguously identified in the tissues of the host rat.



Exp. 3. Defining pattern of tumor entry to the brain.

a. Pattern after 1 day. Our first goal was to confirm that injected tumor cells enter the cerebral vessels in the same pattern as the microspheres (*Exp. 1*, above). Two rats received ten million MATB/lacZ tumor cells, injected into the carotid artery, using the method developed in *Exp. 1*. The rats were sacrificed after one day. Cryostat sections (6u) were taken at regular intervals through the brain. The histochemical stain for b-gal was applied, using the methods developed *Exp. 2* (above).

Results and conclusions. We did observe tumor in brain vessels, in the same general pattern seen with the microspheres. This confirmed that the tumor was being delivered to the cerebral vessels.

b. Pattern after 1 week. A lower dose of MATB/lacZ tumor cells (0.5×10^6) was injected into the carotid artery, following the methods described above, and the rats were sacrificed on day 7. For each rat (n=9), 4 - 5 sections taken through the brain were stained for the b-gal tumor marker, using methods described above.

Findings. Of the 9 rats analyzed, 6/9 showed tumor in the sections analyzed (4-5 sections / rat). The tumor most often appeared as a small ball or nodule of cells in the choroid plexus, most often in the lateral ventricle on the injection side. Minimal tumor was seen away from the ventricle.

Interpretation. We had shown that inert micro-spheres do enter vessels in the brain proper (*Exp. 1*, above), and so does tumor when examined within one day after injection (*a*, above). Re-examination showed that, immediately after injection, some micro-spheres and tumor are also present in the choroid plexus. However, cerebral vessels predominate.

Taken together, these findings imply that the predominance of tumor in the choroid plexus on day 7 reflects preferential retention or growth of the tumor in the choroid plexus, rather than preferential delivery to that site. This may simply reflect preferential retention in the tortuous choroid vessels.

Tumor in the choroid plexus is of interest for some tumors, but rare for breast metastases. In this project, we will focus on tumor that enters the brain proper, as described below.

Exp. 4. Defining pattern of accumulating metastases. Graded numbers of tumor cells (0.5 , 1 , and 2×10^6) were injected as described above. A total of 33 rats were analyzed, divided into 9 experimental groups in 3 experiments. (Within each group, all rats were treated identically, on the same day.)

Rats were examined daily for failure to groom, imbalance or other abnormalities of gait, weakness, lethargy, swollen or crusted eyes or mouth, swollen jaw or lymph node. Failure to groom was the earliest sign of illness; rats were sacrificed as soon any of the other signs appeared (usually within one day).

Brains were analyzed as described above. An overview of the findings for the 33 rats is given below.

Findings.

- a. For the dose range used ($0.5 - 2 \times 10^6$ tumor cells), rats became ill at 9-14 days.
 - b. For each rat, at least 8 slides were analyzed. For 31/33 rats, tumor was detected within the brain. For 1 rats, tumor was seen only in the meninges. For 1 rat, tumor was not detected in the brain, but was detected in the lymph node and lung.
 - c. For the 31 rats with tumor within the brain, all had tumor within the ventricles and also in the brain proper. Some of the tumor in the brain proper appeared to have reached the brain by breaking through the ventricle wall. Other tumor appeared to have entered the brain at cerebral vessels (fig. 2, above). Tumor was also found in the meninges, although that is not our focus in this project.
 - d. The time-to-illness was dose-dependent. Among all rats that received 0.5×10^6 cells, rats became ill at 11-14 days (19 rats, 3 expts). Among all rats that received 1×10^6 cells, rats became ill at 10-11 days (10 rats, 2 expts). Four rats that received 2×10^6 cells all became ill at 9 days (4 rats, 1 exp). In 8/9 experimental groups, the rats within each group became ill on the same day. In the 9th group, the rats became ill within one day of each other.
 - e. The extent of metastatic tumor in the brain proper was dose-dependent. For example, for each rat that showed tumor within in the brain after receiving 0.5 or 1×10^6 cells, we selected the slide with the most tumor. Among all rats receiving 0.5×10^6 cells (17 rats in 5 experimental groups), a mean of 6-11 sites per slide was counted in the different experimental groups. Among all rats receiving 1×10^6 cells (9 rats in 3 experimental groups), a mean of 16-26 sites per slide were counted in the different experimental groups. Further analysis, including additional measures of tumor growth and additional slides, is on-going.
- Summary and conclusions.** Metastatic tumor was found in the brain proper of 31/33 rats. The amount of tumor and the time-to-illness were dose-dependent. Therefore, this model can be used to study blood-borne metastases in the brain. Rats receiving 0.5×10^6 cells survived for at least 10 days, which is enough time for the studies planned in Tasks 3-5.

Exp. 5. Alternative injection routes. In the studies above, metastatic tumor was seen in the brain after the tumor cells had been injected into the carotid artery. To confirm that the intra-carotid injections were an important component of the model, we directly compared other routes.

Rats in the test group received intra-peritoneal (*ip*) injection of 1 or 3×10^6 *MATB/lacZ* tumor cells. As controls, other rats received aliquots of the same cell preparation, delivered into the carotid artery. In a separate study, 2×10^5 tumor cells injected directly into the brain (*intracerebral injection*) was compared to tumor delivered into the carotid artery.

Results and conclusions. The rats receiving *ip* tumor did develop tumor in the periphery, but tumor was rarely seen in the brain. In contrast, the rats that received intra-carotid tumor showed metastatic brain tumor in almost every rat, as described above. This confirms the value of the intra-carotid injections.

Rats receiving direct intracerebral injections of tumor did show tumor in the brain. However, the tumor grew primarily as an expanding mass, confirming our earlier work. This was a clear contrast to the disseminated tumor seen after intra-carotid injection. This confirms the importance of delivering tumor via the blood.

Task 2. Introduce b-gal marker into an alternative cell line (AIM 1B), concurrent with Task 1.

Sub-tasks (summarized). Transduce the alternative cell line with the b-gal marker. Define growth characteristics and compare them to those of the cell line studied in *Task 1*.

We had obtained a "brain-selected" variant of the 13762 rat mammary carcinoma cell line from the late Dr. Peter Steck. To prepare the variant, tumor had been implanted extra-cranially, then harvested from the brain. We introduced the lacZ gene into these cells, and selected subclones with strong, constitutive b-gal expression. The methods were as described in *Task 1, exp. 2* (above). The cells were injected into the carotid artery, and the pattern of metastasis was defined as described in *Task 1*, above.

Results and conclusion. We found that this alternative cell line did not give as many brain mets as the *MATB/lacZ* cell line described in *Task 1*, above. Therefore, for future studies, we will use the *MATB/lacZ* cell line that is characterized in *Task 1*.

Task 3. Use the model to study T cell surveillance (AIM 2), months 7-18.

Sub-tasks (summarized). Inject mammary carcinoma cell line into the blood, and allow mets to accumulate in the brain. Introduce tumor-specific T cells into the blood. Give intra-cerebral injections of cytokines that are known to enhance T cell entry from blood and/or activate antigen presenting cells (APC) in the brain. Determine if the injected cytokine increases the % of metastases that have adjacent T cells, and/or the % of metastases that have adjacent activated APC.

Exp. 1. Choice of conditions for cytokine injection. Our prior work had shown that intra-cerebral injection of either IFN-g or TNF can enhance T cell entry to the brain. In preparation for our studies here, we extended our earlier work: We defined the dose-response curves for each cytokine. We directly compared TNF-a alone, IFN-g alone, and both together.

As shown in our own work and many others' (Lampson 98), a T cells is more likely to enter the brain if it has been activated by exposure to its cognate antigen. Therefore, the first step in these experiments was to create a pool of activated T cells, by immunizing the rats with an appropriate antigen. In most experiments, we immunized the rats with b-gal, which serves as a defined tumor antigen in our mammary carcinoma cell lines (as described in *Task 1, exp. 2*). For comparison, in some studies we immunized rats with the normal CNS antigen, myelin basic protein (MBP).

To immunize the rats, the antigen of study (b-gal protein or MBP) was emulsified in Complete Freund's Adjuvant (CFA), and injected into multiple sites under the skin, following common procedures. Two weeks later, the test cytokine (TNF-a, IFN-g or TNF+IFN) was injected stereotactically into the striatum. Preliminary work had shown that T cell numbers peak two days after cytokine injection. Therefore, rats were sacrificed two days after injection of TNF-a, IFN-g or TNF+IFN. Controls received vehicle instead of cytokine.

Cryostat sections through the injection region were stained to reveal T cells, using the pan T cell monoclonal antibody, R73, in the ABC reaction with DAB substrate. The density of T cells in the perivascular space (PVS) and T cells in the brain proper were counted in a standardized region of interest, with the aid of an eye-piece grid. The individual experiments are summarized below.

a. We defined the dose-response curve for the effect of injected TNF-a on T cell entry. Rats received graded doses of TNF-a, and T cells were counted in the perivascular space and brain parenchyma proper. Three regions were counted separately: cerebral cortex, corpus callosum, and striatum.

b. We defined the dose-response curve for IFN-a. Methods were as in **a**.

c. We defined the effect of combining TNF-a with IFN-g. We worked from the dose-response curves defined in **a** and **b**. We selected a dose of TNF-a at the beginning of the plateau portion of the curve, where raising the dose of TNF-a alone gave little further increase in T cell entry. We selected a dose of IFN-g that, by itself, had a minimal effect on T cell entry.

d. In parallel assays, adjacent slides were stained with monoclonal antibody OX6 to reveal potential antigen-presenting cells (APC, including microglia and other phagocytes) with strong class II MHC antigen expression, which is commonly taken as a sign of activation.

Findings and conclusions. IFN-g alone and TNF-a alone each enhanced T cell entry to the brain. However, IFN-g was more effective at activating APC. Therefore, we will begin with IFN-g. Although APC were activated at a relatively low dose (100 U IFN-g), higher doses were need to achieve optimal T cell entry. Therefore, we will begin with a dose of 1000 U IFN-g.

Although IFN-g alone (or TNF-a alone) can increase T cell entry, the efficacy is different in different brain regions. Injecting [TNF-a + IFN-g] gives a more uniform T cell entry. If we find that IFN-g alone does not increase T cell interactions with brain mets in all regions, we will compare the effects of [TNF-a + IFN-g].

Exp. 2 Controls: Effects of needle wound, and effects of IFN-g without T cell activation. Our planned therapy has two steps: 1) Increase the number of activated tumor-specific T cells in the blood. 2) Inject cytokine intracerebrally, to enhance T cell entry to the brain. To correctly interpret the results:

- a) We needed to define how an intracerebral needle wound might affect the pattern of metastasis.
- b) We needed to define how injection of IFN-g might affect metastases, even if T cells were not deliberately activated.

Methods. Rats received 0.5×10^6 MATB/lacZ cells in the carotid artery, using the methods of Task 1.

The rats were divided into 3 groups. Group 1 received 1000 U IFN-g, injected stereotactically into the striatum, 5 day after tumor injection. However, no effort was made to activate T cells.

Group 2 received vehicle instead of IFN-g. Group 3 received no treatment.

Rats were sacrificed at the first signs of illness, as described above. Sites of metastatic tumor away from the ventricle were measured as described above.

Results and follow-up.

Rats receiving no treatment (n=3) became ill on day 12 (3/3), and had a mean of 11 tumor sites away from the ventricle (per slide).

Rats that receives buffer injection (n=4) became ill on day 11 (4/4) and had a mean of 11 tumor sites away from the ventricle (per slide). Thus, the number of tumor sites was not dramatically increased or decreased by making a needle wound in the brain, 5 days after tumor injection.

Rats that received IFN-g (n = 4) became ill on day 12 (4/4) and had 10 tumor sites per slide. Thus, the number of tumor sites was not dramatically increased or decreased by the injection of IFN-g 5 days after tumor injection.

We are now continuing the analysis: We will analyze additional slides. We will compare different measures of tumor growth: For example, we will also measure the total amount of metastatic tumor, and the size of individual mets.

Taken together, this will define how a needle wound alone, and how injection of IFN-g alone (without T cell activation) affect the pattern of metastases, when the IFN-g is injected 5 days after tumor has been placed in the blood.

Exp. 3. Is the IFN-g still active in the presence of growing tumor? It is well known that tumors can be immunosuppressive. However, "suppression" covers many functions. We have argued that it is important to define "suppression" in terms of a specific function and site (Lampson 98, 02).

- a. We have shown that IFN-g is able to activate antigen presenting cells in the presence of growing brain tumor; this particular function of IFN-g is not suppressed (Dutta et al 98).

- b. We went on to ask if IFN-g can also increase entry of T cells to the brain, in the presence of growing tumor.

These studies were conducted in parallel with the tasks above, and used an earlier tumor model. In this model, tumor was implanted in the brain, rather than entering through the blood. Analysis focused on small tumor masses (*micro-tumor*) that had spread away from the site of implantation.

Tumor-bearing rats received injections of IFN-g or vehicle control. The number of micro-tumors with adjacent T cells, and the number of T cells per micro-tumor were counted. The Student t test was used to compare the mean values between groups.

Findings. In 3/3 experiments, IFN-g significantly increased the number of T cells per micro-tumor ($p < 0.05, 0.01, 0.01$, respectively).

Conclusion and follow-up. Taken together, a. and b. support our hypothesis that IFN-g can still display the activities of interest (activation of APC, enhanced T cell entry) in the presence of growing tumor. Now we will test this directly in our metastatic tumor model.

Exp. 4. Selecting the T cell source. The first step in T cell-mediated therapy is to stimulate T cells (outside the brain), as discussed above. This can be achieved by immunizing the patient or tumor-bearing rat directly. Or it can be achieved by adoptive transfer of T cells from culture. We have now developed methods that make adoptive transfer the procedure of choice for this Task, as described below.

T cell lines were raised against our model tumor antigen, b-gal, using standard methods.

Recently-stimulated T cells were labeled with the fluorescent dye, 5-(and-6)chloromethyl(benzoyl)amino) tetramethylrhodamine (CMTMR) (Molecular Probes, Eugene, OR).

The T cells were injected into the tail vein of rats with established tumor in the brain, using our preliminary tumor model (*Exp. 3*).

To increase the efficiency of T cell entry (above), IFN-g was injected stereotactically near the tumor site.

The optimal timing between IFN-g injection and T cell injection was determined: Groups of tumor-bearing rats received stereotactic injection of IFN-g on different days, days “- 2”, “-1”, and “0”, respectively.

Recently stimulated, fluorescence-labeled T cells (*fl-T cells*) were injected into the lateral tail vein of all rats on “day 0.” All rats were sacrificed 1 day later. Sections were cut through the tumor region. Slides were stained histochemically to reveal the b-gal+ tumor. Slides were also examined by fluorescence microscopy, to reveal the presence of the fl-T cells.

Results and conclusions. The most fl-T cells were seen in rat that had received IFN-g one day before T cells. Therefore, this schedule was chosen for later experiments.

The fl-T cells were always associated with tumor; they were not seen in tumor-free areas.

Value for this project. As a source of activated T cells, intravenous injection of fl-T cells offers several advantages over immunization of the tumor-bearing rat. Aliquots from the same T cell population can be delivered to all experimental groups. The fluorescent label allows unambiguous identification of cells that have been activated against tumor antigen. The cells can be labeled with different fluorochromes, allowing direct comparison of two populations within the tumor-bearing host. For example, we can compare tumor-specific T cells vs. control T cells, by labeling them with different dyes.

Exp. 5: Test of hypothesis. The work above lays the foundation for testing our *hypothesis* that intracerebral IFN-g can enhance T cell/tumor interactions. The final experiment will be:

1. Establish metastatic tumor, using methods developed in *Task 1* and tumor cell line chosen in *Task 2*.
2. Inject IFN-g stereotactically into the brain, using doses chosen in *Task 3*.
3. Inject stimulated fl-T cells into lateral tail vein, using methods developed in *this task, exp. 3* and following schedule selected in *this task, exp. 3*.
4. Sacrifice rats. Identify tumor by histochemical stain for b-gal, fl-T cells by fluorescence microscopy, and antigen-presenting cells by staining with monoclonal antibody OX6, using methods described above.
5. Measure numbers of metastatic tumor cells that have adjacent fl-T cells and/or adjacent activated (OX6+) APC. Compare IFN-treated rats to controls.
6. Apply appropriate tests of statistical significance.

Task 4. Define therapy, (AIM 2) months 7-30 (overlaps Tasks 3 and 5).

Sub-tasks (summarized). Treat tumor-bearing rats as in *Task 3*, but sacrifice at progressively longer intervals. Evaluate efficacy and safety of cytokine-enhanced T cell attack.

Task 3 provides the necessary background for this work. As we obtain short-term results in *Task 3*, we will follow efficacy and safety in longer-term follow-up studies.

Task 5. Evaluate Prophylaxis (AIM 2C), months 24-36 (overlaps Task 4).

Sub-tasks (summarized). *Task 4* (above) evaluates therapy that is given *after* mets have entered the brain. The aim in *Task 5* is to evaluate prophylaxis, when therapy is given *before* mets enter the brain. In accordance with the approved schedule, the major work on this task will begin after more data from *Task 4* has been obtained.

KEY RESEARCH ACCOMPLISHMENTS

- Validated methods for intracarotid injection.
- Developed brain-metastasizing mammary carcinoma cell line with b-gal marker.
Marker allows identification of tumor cells.
Also serves as defined tumor antigen.
- Showed that, after intracarotid injection, tumor produces brain mets.
Amount of tumor and time-to-illness are dose-dependent.
Time to illness is sufficient for planned studies.
- Verified choice of IFN-g as the immuno-activating cytokine.
- Confirmed IFN-g is still active in presence of growing tumor.
- Developed fluorescence-labeled T cell lines against the tumor antigen

REPORTABLE OUTCOMES

Abstracts.

Kapoor R, Durham J, Kondo Y, Lampson LA. Distribution of brain-metastasizing mammary carcinoma after intracarotid artery delivery in the rat. AACR Annual Meeting, 2002, submitted.

Kondo Y, Lampson LA. Enhancing T cell surveillance of brain micro-tumor: Different drugs optimize T cell entry to grey or white matter. Proc. AACR. 2000: 41, 192, abstract #1226.

Lampson LA, McCluskey LP, Kondo Y, Kapoor R, McCormack SE, Seabrook TJ, Preidis GA, Dube C, Dutta T. Micro-tumor immunotherapy: Beyond vaccines. 6th Int. Congress of Neuroimmunology, Edinburgh, September, 2001. J. Neuroimmunol 2001, in press.

Seabrook TJ, Lampson LA. IFN- γ increases the entry of T cells at micro-tumors in a rat model of brainstem glioma. Abstract submitted for AAI Annual Mtg, New Orleans, April, 2002.

Presentation (in addition to meeting abstracts listed).

The PI presented information about the patterns of metastasis to brain at the Glioma Invasion Forum, Ghent, Belgium, November, 2001.

Cell line. The MATB/lacZ cell line metastasizes to the brain and constitutively expresses the lacZ gene product, which serves as a tumor marker and a defined tumor antigen.

Manuscripts in preparation.

Kondo Y, Lampson, LA: Distinct patterns of enhanced T cell surveillance and microglial activation after intracerebral cytokine injection. In revision.

Seabrook TJ, Lampson LA. Enhanced T cell surveillance in the brain after intracerebral injection of IFN-g: In preparation.

CONCLUSIONS

We have developed a novel experimental model that is appropriate for the study of blood-borne breast cancer metastasis to the brain.

We have laid the foundation for testing our hypothesis that T cells can be used to deliver therapy to metastatic breast cancer in the brain. We developed fluorescence-labeled T cell lines, and defined conditions for their use. We defined conditions for use of IFN-g as an immune-activating cytokine, and showed that it retains its activity in the presence of growing tumor.

Why this is important. Metastasis to the brain is an important and growing cause of mortality for breast cancer patients. Novel therapies, and appropriate experimental models to test the therapies, were both needed. Our novel model is appropriate for our uses, and of general interest. The background work we have done will permit a well-controlled test of our hypothesis: That T cells can be made to deliver therapy to metastatic breast cancer in the brain.

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